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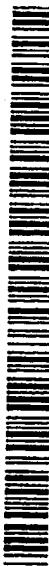
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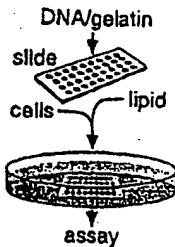
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(54) Title: REVERSE TRANSFECTION METHOD



(57) Abstract: A reverse transfection method of introducing DNA of interest into cells and arrays, including microarrays, of reverse transfected cells.

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## REVERSE TRANSFECTION METHOD

### BACKGROUND OF THE INVENTION

Genome and expressed sequence tag (EST) projects are rapidly cataloging and cloning the genes of higher organisms, including humans. The emerging challenge is to uncover the functional roles of the genes and to quickly identify gene products with desired properties. The growing collection of gene sequences and cloned cDNAs demands the development of systematic and high-throughput approaches to characterizing the gene products. The uses of DNA microarrays for transcriptional profiling and of yeast two-hybrid arrays for determining protein-protein interactions are recent examples of genomic approaches to the characterization of gene products (Schena, M., *et al.*, *Nature*, 10:623 (2000)). Comparable strategies do not exist to analyze the function, within mammalian cells, of large sets of genes. Currently, *in vivo* gene analysis can be done --on a gene-by-gene scale-- by transfecting cells with a DNA construct that directs the overexpression of the gene product or inhibits its expression or function. The effects on cellular physiology of altering the level of a gene product is then detected using a variety of functional assays.

A variety of DNA transfection methods, such as calcium phosphate coprecipitation, electroporation and cationic liposome-mediated transfection (e.g.,

lipofection) can be used to introduce DNA into cells and are useful in studying gene regulation and function. Additional methods, particularly high throughput assays that can be used to screen large sets of DNAs to identify those encoding products with properties of interest, would be useful to have available.

## 5 SUMMARY OF THE INVENTION

Described herein is a strategy for the high throughput analysis of gene function in mammalian cells. A method to create transfected cell microarrays that are suitable for rapidly screening large sets of cDNAs or DNA constructs for those encoding desired products or for causing cellular phenotypes of interest is described.

- 10 Using a slide printed with sets of cDNAs in expression vectors, a living microarray of cell clusters expressing the gene products has been generated. The cell clusters can be screened for any property detectable on a surface and the identity of the responsible cDNA(s) determined from the coordinates of the cell cluster with a phenotype of interest.
- 15 Accordingly, the present invention relates to a method, referred to as a reverse transfection method, in which a defined nucleic acid (a nucleic acid of known sequence or source), also referred to as a nucleic acid of interest or a nucleic acid to be introduced into cells, is introduced into cells in defined areas of a lawn of eukaryotic cells, in which it will be expressed or will itself have an effect on or
- 20 interact with a cellular component or function. Any suitable nucleic acid such as an oligonucleotide, DNA and RNA can be used in the methods of the present invention. The particular embodiments of the invention are described in terms of DNA. However, it is to be understood that any suitable nucleic acid is encompassed by the present invention.
- 25 In one embodiment, the present invention relates to a method in which defined DNA (DNA of known sequence or source), also referred to as DNA of interest or DNA to be introduced into cells, is introduced into cells in defined areas of a lawn of eukaryotic cells, in which it will be expressed or will itself have an effect on or interact with a cellular component or function. In the method, a mixture,
- 30 defined below, comprising DNA of interest (such as cDNA or genomic DNA incorporated in an expression vector) and a carrier protein is deposited (e.g., spotted

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or placed in small defined areas) onto a surface (e.g., a slide or other flat surface, such as the bottoms of wells in a multi-welled plate) in defined, discrete (distinct) locations and allowed to dry, with the result that the DNA-containing mixture is affixed to the surface in defined discrete locations.

5        Such locations are referred to herein, for convenience, as defined locations. The DNA-containing mixture can be deposited in as many discrete locations as desired. The resulting product is a surface bearing the DNA-containing mixture in defined discrete locations; the identity of the DNA present in each of the discrete locations (spots) is known/defined. Eukaryotic cells, such as mammalian cells (e.g.,  
10      human, monkey, canine, feline, bovine, or murine cells), bacterial, insect or plant cells, are plated (placed) onto the surface bearing the DNA-containing mixture in sufficient density and under appropriate conditions for introduction/entry of the DNA into the eukaryotic cells and expression of the DNA or its interaction with cellular components. Preferably, the eukaryotic cells (in an appropriate medium)  
15      are plated on top of the dried DNA-containing spots at high density (e.g., 1 x 10<sup>5</sup>/cm<sup>2</sup>), in order to increase the likelihood that reverse transfection will occur. The DNA present in the DNA-containing mixture affixed to the surface enters eukaryotic cells (reverse transfection occurs) and is expressed in the resulting reverse transfected eukaryotic cells.

20       In one embodiment of the method, referred to as a "gelatin-DNA" embodiment, the DNA-containing mixture, referred to herein as a gelatin-DNA mixture, comprises DNA (e.g., DNA in an expression vector) and gelatin, which is present in an appropriate solvent, such as water or double deionized water. The mixture is spotted onto a surface, such as a slide, thus producing a surface bearing  
25      (having affixed thereto) the gelatin -DNA mixture in defined locations. The resulting product is allowed to dry sufficiently that the spotted gelatin -DNA mixture is affixed to the slide and the spots remain in the locations to which they have become affixed, under the conditions used for subsequent steps in the method. For example, a mixture of DNA in an expression vector and gelatin is spotted onto a  
30      slide, such as a glass slide coated with Σ poly-L-lysine (e.g., Sigma, Inc.), for example, by hand or using a microarrayer. The DNA spots can be affixed to the slide by, for example, subjecting the resulting product to drying at room temperature, at

elevated temperatures or in a vacuum-dessicator. The length of time necessary for sufficient drying to occur depends on several factors, such as the quantity of mixture placed on the surface and the temperature and humidity conditions used.

The concentration of DNA present in the mixture will be determined

- 5 empirically for each use, but will generally be in the range of from about 0.01  $\mu\text{g}/\mu\text{l}$  to about 0.2  $\mu\text{g}/\mu\text{l}$  and, in specific embodiments, is from about 0.02  $\mu\text{g}/\mu\text{l}$  to about 0.10  $\mu\text{g}/\mu\text{l}$ . Alternatively, the concentration of DNA present in the mixture can be from about 0.01  $\mu\text{g}/\mu\text{l}$  to about 0.5  $\mu\text{g}/\mu\text{l}$ , from about 0.01  $\mu\text{g}/\mu\text{l}$  to about 0.4  $\mu\text{g}/\mu\text{l}$  and from about 0.01  $\mu\text{g}/\mu\text{l}$  to about 0.3  $\mu\text{g}/\mu\text{l}$ . Similarly, the concentration  
10 of gelatin, or another carrier macromolecule, can be determined empirically for each use, but will generally be in the range of 0.01% to 0.5% and, in specific  
embodiments, is from about 0.05% to about 0.5%, from about 0.05% to about 0.2%  
or from about 0.1% to about 0.2%. The final concentration of DNA in the mixture  
(e.g., DNA in gelatin) will generally be from about 0.02  $\mu\text{g}/\mu\text{l}$  to about 0.1  $\mu\text{g}/\mu\text{l}$   
15 and in a specific embodiment described herein, DNA is diluted in 0.2% gelatin  
(gelatin in water) to produce a final concentration of DNA equal to approximately  
0.05  $\mu\text{g}/\mu\text{l}$ .

If the DNA used is present in a vector, the vector can be of any type, such as a plasmid or viral-based vector, into which DNA of interest (DNA to be expressed in  
20 reverse transfected cells) can be introduced and expressed (after reverse transfection) in recipient cells. For example, a CMV-driven expression vector can be used.

Commercially available plasmid-based vectors, such as pEGFP (Clontech) or  
pcDNA3 (Invitrogen), or viral-based vectors can be used. In this embodiment, after  
drying of the spots containing the gelatin-DNA mixture, the surface bearing the  
25 spots is covered with an appropriate amount of a lipid-based transfection reagent and  
the resulting product is maintained (incubated) under conditions appropriate for  
complex formation between the DNA in the spots (in the gelatin-DNA mixture) and  
the lipid-based transfection reagent. In one embodiment, the resulting product is  
incubated for approximately 20 minutes at 25°C. Subsequently, transfection reagent  
30 is removed, producing a surface bearing DNA (DNA in complex with transfection  
reagent), and cells in an appropriate medium are plated onto the surface. The

resulting product (a surface bearing DNA and plated cells) is maintained under conditions that result in entry of the DNA into plated cells.

A second embodiment of the method is referred to as a "lipid -DNA" embodiment. In this embodiment, a DNA-containing mixture (referred to herein as a 5 lipid-DNA mixture) which comprises DNA (e.g., DNA in an expression vector); a carrier protein (e.g., gelatin); a sugar, such as sucrose; a buffer that facilitates DNA condensation and an appropriate lipid-based transfection reagent is spotted onto a surface, such as a slide, thus producing a surface bearing the lipid-DNA mixture in defined locations. The resulting product is allowed to dry sufficiently that the 10 spotted lipid-DNA mixture is affixed to the slide and the spots remain in the locations to which they have become affixed, under the conditions used for subsequent steps in the method. For example, a lipid-DNA mixture is spotted onto a slide, such as a glass slide coated with  $\Sigma$  poly-L-lysine (e.g., Sigma, Inc.), for example, by hand or using a microarrayer. The DNA spots can be affixed to the 15 slide as described above for the gelatin-DNA method.

The concentration of DNA present in the mixture will be determined empirically for each use, but will generally be in the range of 0.5  $\mu\text{g}/\mu\text{l}$  to 1.0  $\mu\text{g}/\mu\text{l}$ . A range of sucrose concentrations can be present in the mixture, such as from about 0.1M to about 0.4M. Similarly, a range of gelatin concentrations can be present in 20 the mixture, such as from about 0.01% to about 0.05%. In this embodiment, the final concentration of DNA in the mixture will vary and can be determined empirically. In specific embodiments, final DNA concentrations range from about 0.1  $\mu\text{g}/\mu\text{l}$  to about 2.0  $\mu\text{g}/\mu\text{l}$ . If a vector is used in this embodiment, it can be any vector, such as a plasmid, or viral-based vector, into which DNA of interest (DNA to 25 be expressed in reverse transfected cells) can be introduced and expressed (after reverse transfection), such as those described for use in the gelatin-DNA embodiment.

After drying is complete (has occurred to a sufficient extent that the DNA remains affixed to the surface under the conditions used in the subsequent steps of 30 the method), eukaryotic cells into which the DNA is to be reverse transfected are placed on top of the surfaces onto which the DNA-containing mixture has been affixed. Actively growing cells are generally used and are plated, preferably at high

density (such as  $1 \times 10^5/\text{cm}^2$ ), on top of the surface containing the affixed DNA-containing mixture in an appropriate medium, such as Dulbecco's Modified Eagles Medium (DMEM) containing 10% heat-inactivated fetal serum (IFS) with L-glutamine and penicillin/streptomycin (pen/strep). Other media can be used and

5 their components can be determined based on the type of cells to be transfected. The resulting slides, which contain the dried lipid-DNA mixture and cells into which the DNA is to be reverse transfected, are maintained under conditions appropriate for growth of the cells and entry of DNA, such as an entry of an expression vector containing the DNA, into cells. In the present method, approximately one to two

10 cell cycles are sufficient for reverse transfection to occur, but this will vary with the cell type and conditions used and the appropriate length of time for a specific combination can be determined empirically. After sufficient time has elapsed, slides are assessed for reverse transfection (entry of DNA into cells) and expression of the encoded product or effect of the introduced DNA on reverse-transfected cells, using

15 known methods. This can be done, for example, by detecting immunofluorescence or enzyme immunocytochemistry, autoradiography, in situ hybridization or other means of detecting expression of the DNA or an effect of the encoded product or of the DNA itself on the cells into which it is introduced. If immunofluorescence is used to detect expression of an encoded protein, an antibody that binds the protein

20 and is fluorescently labeled is used (e.g., added to the slide under conditions suitable for binding of the antibody to the protein) and the location (spot or area of the surface) containing the protein is identified by detecting fluorescence. The presence of fluorescence indicates that reverse transfection has occurred and the encoded protein has been expressed in the defined location(s) which show fluorescence. The

25 presence of a signal, detected by the method used, on the slides indicates that reverse transfection of the DNA into cells and expression of the encoded product or an effect of the DNA in recipient cells has occurred in the defined location(s) at which the signal is detected. As described above, the identity of the DNA present at each of the defined locations is known; thus, when expression occurs, the identity of the

30 expressed protein is also known.

Thus, the present invention relates, in one embodiment, to a method of expressing defined DNA, such as cDNA or genomic DNA, in defined locations or

areas of a surface onto which different DNAs, such as DNA in a vector, such as an expression vector, has been affixed, as described herein. Because each area of the surface has been covered/spotted with DNA of known composition, it is a simple matter to identify the expressed protein. In addition, the present method is useful to

5 identify DNAs whose expression alters (enhances or inhibits) a pathway, such as a signaling pathway in a cell or another property of a cell, such as its morphology or pattern of gene expression. The method is particularly useful, for example, as a high-throughput screening method, such as in a microarray format. It can be used in this format for identifying DNAs whose expression changes the phosphorylation

10 state or subcellular location of a protein of interest or the capacity of the cell to bind a reagent, such as a drug or hormone ligand. In a second embodiment, which is also useful as a high-throughput screening method, DNA reverse transfected into cells has an effect on cells or interacts with a cellular component(s) without being expressed, such as through hybridization to cellular nucleic acids or through

15 antisense activity.

Also the subject of this invention are arrays, including microarrays, of defined DNAs spotted onto (affixed to) a surface and array: including microarrays of reverse transfected cells spotted to (affixed to) a surface by the method described herein. Such arrays can be produced by the gelatin-DNA embodiment or the lipid-DNA embodiment of the present method. Arrays of this invention are surfaces, such as slides (e.g., glass or  $\Sigma$  poly-L-lysine coated slides) or wells, having affixed thereto (bearing) in discrete, defined locations DNAs, such as cDNAs or genomic DNA, or cells containing DNA of interest introduced into the cells by the reverse transfection method described herein.

25 A method of making arrays of the present invention is also the subject of this invention. The method comprises affixing DNAs or reverse transfected cells onto a surface by the steps described herein for the gelatin-DNA embodiment or the lipid-DNA embodiment.

A DNA array of the present invention comprises a surface having affixed thereto, in discrete, defined locations, DNA of known sequence or source by a method described herein. In one embodiment, DNA is affixed to a surface, such as a slide, to produce an array (e.g., a macro-array or a micro-array) by spotting a gelatin-

DNA mixture, as described herein, onto the surface in distinct, defined locations (e.g., by hand or by using an arrayer, such as a micro-arrayer) and allowing the resulting surface bearing the gelatin-DNA mixture to dry sufficiently that the spots remain affixed to the surface under conditions in which the arrays are used. In an 5 alternative embodiment, DNA is affixed to a surface, such as a slide, to produce an array by spotting a lipid-DNA mixture, as described herein, onto the surface in distinct defined locations (e.g., by hand or by using an arrayer, such as a micro- arrayer) and allowing the resulting surface bearing the lipid-DNA mixture to dry sufficiently that the spots remain affixed to the surface under the conditions in which 10 the arrays are used. This result in production of a surface bearing (having affixed thereto) DNA-containing spots.

An array of reverse transfected cells can also be produced by either embodiment described herein. In the gelatin-DNA embodiment, the steps described above for producing DNA arrays are carried out and subsequently, the surface 15 bearing the DNA-containing spots is covered with an appropriate amount of a lipid-based transfection reagent and the resulting product is maintained (incubated) under conditions appropriate for complex formation between DNA in the spots and the reagent. After sufficient time (e.g., about 20 minutes at 25°C) for complex formation to occur, transfection reagent is removed, producing a surface bearing 20 DNA and cells in an appropriate medium are added. The resulting product (a surface bearing DNA and plated cells) is maintained under conditions that result in entry of DNA into plated cells, thus producing an array (a surface bearing an array) of reverse transfected cells that contain defined DNA and are in discrete, defined locations on the array. Such cell arrays are the subject of this invention.

25 In the lipid-DNA embodiment, the steps described above for producing DNA arrays are carried out and subsequently (after drying is sufficient to affix the DNA-containing spots to the surface, such as a slide or well bottom), cells are plated on top of the surface bearing the DNA-containing spots and the resulting slides, which contain the dried lipid-DNA mixture and cells to be reverse transfected, are 30 maintained under conditions appropriate for growth of the cells and entry of DNA into the cells, thus producing an array (a surface bearing an array) of reverse

transfected cells that contain defined DNA and are in discrete, defined locations on the array. Such arrays are the subject of this invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color.

5 Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1 is a schematic representation of one embodiment of the present method of reverse transfection, in which cDNA (HA-GST, HA-FKBP12 or myc-FRB) in an expression vector (prk5) was introduced into cells by the following  
10 procedures: combining cDNA in an expression vector, a lipid-based transfection reagent and a carrier protein, to produce a mixture; spotting the mixture onto a glass slide; allowing the spotted mixture to dry on the slide surface; plating human embryonic kidney (HEK 293T) cells into which cDNA is to be introduced onto the slide; maintaining the resulting slide under conditions appropriate for reverse  
15 transfection to occur; and detecting immunofluorescence using a fluorescently labeled antibody that binds HA but not myc, demonstrating the presence and location of expressed cDNA.

Figure 2 shows the results of reverse transfection of HEK293T cells with HA-GST, as demonstrated using anti-HA immunofluorescence.

20 Figure 3 shows the results of reverse transfection of HEK293T cells with pBABE EGFP, as demonstrated by detecting endogenous fluorescence of EGFP.

Figure 4A is a schematic for making transfected cell microarrays using a well-less transfection of plasmid DNAs in defined areas of a lawn of mammalian cells. Plasmid DNA dissolved in an aqueous gelatin solution is printed on a glass  
25 slide using a robotic arrayer. The slide is dried and the printed array covered with a lipid transfection reagent. After removal of the lipid, the slide is placed in a culture dish and covered with cells in media. The transfected cell microarray forms in 1-2 days and is then ready for downstream assays. An alternative method in which the lipid is added to the DNA/gelatin solution prior to printing is also described.

30 Figure 4B is a GFP-expressing microarray made from a slide printed in a 12 x 8 pattern with a GFP expression construct.

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Figure 4C is a higher magnification image obtained with fluorescence microscopy of the cell cluster boxed in Figure 4B. Scale bar equals 100  $\mu\text{m}$ .

Figure 4D is a graph of GFP cDNA (picograms) versus mean signal intensity +/- S.D. showing expression levels of clusters in a transfected cell microarray are proportional, over a four-fold range, to the amount of plasmid DNA printed on the slide. Arrays were printed with elements containing the indicated amounts of the GFP construct. Amount of DNA assumes a one nanoliter printing volume. After transfection, the mean +/- S.D. of the fluorescence intensities of the cell clusters were determined. Arrays were prepared as described in Example 3 except that the concentration of the GFP expression plasmid was varied from 0.010-0.050  $\mu\text{g}/\mu\text{l}$  while the total DNA concentration was kept constant at 0.050  $\mu\text{g}/\mu\text{l}$  with empty vector (prk5). Cell clusters were photographed and the signal intensity quantitated with Image Quant (Fuji). The fluorescent image is from a representative experiment.

Figure 4E is a scan image showing that by printing mixtures of two plasmids, cotransfection is possible with transfected cell microarrays. Arrays with elements containing expression constructs for HA-GST, GFP or both were transfected and processed for anti-myc immunofluorescence. For immunofluorescence staining the cells were fixed as described in Example 3, permeabilized in 0.1% Triton X-100 in PBS for 15 minutes at room temperature and probed with primary and secondary antibodies as described. Primary antibodies were used for 1 hour at room temperature at the following concentrations: 1:500 anti-HA ascites (BaBCo), 2  $\mu\text{g}/\text{ml}$  anti-myc 9E-10 (Calbiochem), 2  $\mu\text{g}/\text{ml}$  anti-V5 (Invitrogen), or 10  $\mu\text{g}/\text{ml}$  4G10 anti-phosphotyrosine (Upstate Biotechnologies). The secondary antibody used was Cy3  $\mu\text{g}/\text{ml}$  labeled anti-mouse antibody (Jackson ImmunoResearch) at 3.1  $\mu\text{g}/\text{ml}$  for 40 minutes at room temperature. Panels labeled Cy3 and GFP show location of clusters expressing HA-GST and GFP, respectively. Merged panel shows superimposition of Cy3 and GFP signals and yellow color indicates co-expression. Scale bar equals 100  $\mu\text{m}$ .

Figure 4F is an enlarged view of boxed area of scan image from Figure 4E.  
30 Figure 5A is a laser scan showing detection of the receptor for FK506. Arrays with elements containing expression constructs for GFP, myc-FKBP12 or

both were printed and transfected with HEK293 cells. 5nM dihydro-FK506 [propyl-<sup>3</sup>H] (NEN) was added to the culture media 1 hour prior to fixation and processing for immunofluorescence and autoradiography. Slides were process for anti-myc immunofluorescence, scanned at 5 μm resolution and photographed using 5 a fluorescent microscope, and then exposed to tritium sensitive film (Hyperfilm, Amersham) for 4 days. Autoradiographic emulsion was performed as described by the manufacturer (Amersham). Laser scans show expression pattern of GFP and FKBP12 and superimposition of both (merged). Film autoradiography detects binding of tritiated FK506 to the same array (autorad film).

10 Figure 5B is a higher magnification image obtained by fluorescent microscopy of an FKBP12-expressing cluster (FKBP12). Emulsion autoradiography detects, with cellular resolution, binding of tritiated FK506 to the same cluster (autorad emulsion).

Figure 5C is a scan showing detected components of tyrosine kinase 15 signaling cascades. 192 V5-epitope-tagged cDNAs in expression vectors were printed in two 8 x 12 subgrids named array 1 and 2. For ease of determining the coordinates of cell clusters within the arrays a border around each array was printed with the GFP expression construct. After transfection, separate slides were processed for anti-V5 or anti-phosphotyrosine immunofluorescence and Cy3 and 20 GFP fluorescence detected. Merged images of array 1 show location of clusters expressing V5-tagged proteins (left panel) and having increased levels of phosphotyrosine (right panel). No DNA was printed in coordinates F10-12.

Figure 5D show two examples of the morphological phenotypes detectable in the transfected cell microarrays described in Figure 5C. Clusters shown are E8 and 25 F7 from array 2.

#### DETAILED DESCRIPTION OF THE INVENTION

A microarray-based system was developed to analyze the function in mammalian cells of many genes in parallel. Mammalian cells are cultured on a glass slide printed in defined locations with solutions containing different DNAs. Cells 30 growing on the printed areas take up the DNA, creating spots of localized transfection within a lawn of non-transfected cells. By printing sets of

complementary DNAs (cDNAs) cloned in expression vectors, micorarrays which comprise groups of live cells that express a defined cDNA at each location can be made. Transfected cell microarrays can be of broad utility for the high-throughput expression cloning of genes, particularly in areas such as signal transduction and  
5 drug discovery. For example, as shown herein, transfected cell microarrays can be used for the unambiguous identification of the receptor for the immunosuppressant FK506 and components of tyrosine kinase pathways.

The present invention relates to a method of introducing defined DNAs into cells at specific discrete, defined locations on a surface by means of a reverse  
10 transfection method. That is, the present method makes use of DNAs, of known sequence and/or source, affixed to a surface (DNA spots), such as a slide or well bottom, and growing cells that are plated onto the DNA spots and maintained under conditions appropriate for entry of the DNAs into the cells. The size of the DNA spots and the quantity (density) of the DNA spots affixed to the surface can be  
15 adjusted depending on the conditions used in the methods. For example, the DNA spots can be from about 100  $\mu\text{m}$  to about 200  $\mu\text{m}$  in diameter and can be affixed from about 200  $\mu\text{m}$  to about 500  $\mu\text{m}$  apart on the surface. The present method further includes identification or detection of cells into which DNA has been reverse transfected. In one embodiment, DNA introduced into cells is expressed in the cells,  
20 either by an expression vector containing the DNA or as a result of integration of reverse transfected DNA into host cell DNA, from which it is expressed. In an alternative embodiment of the present method, DNA introduced into cells is not expressed, but affects cell components and/or function itself. For example, antisense DNA can be introduced into cells by this method and affect cell function. For  
25 example, a DNA fragment which is anti-sense to an mRNA encoding a receptor for a drug can be introduced into cells via reverse transfection. The anti-sense DNA will decrease the expression of the drug receptor protein, causing a decrease in drug binding to cells containing the anti-sense DNA. In the method, a mixture comprising DNA of interest (such as cDNA or genomic DNA incorporated in an expression  
30 vector) and a carrier protein is deposited (e.g., spotted or placed in small defined areas) onto a surface (e.g., a slide or other flat surface, such as the bottoms of wells in a multi-welled plate) in defined, discrete (distinct) locations and allowed to dry,

with the result that the DNA-containing mixture is affixed to the surface in defined discrete locations.

Detection of effects on recipient cells (cells containing DNA introduced by reverse transfection) can be carried out by a variety of known techniques, such as 5 immunofluorescence, in which a fluorescently labeled antibody that binds a protein of interest (e.g., a protein thought to be encoded by a reverse transfected DNA or a protein whose expression or function is altered through the action of the reverse transfected DNA) is used to determine if the protein is present in cells grown on the DNA spots.

10 The nucleic acid used in the methods of the present invention can be oligonucleotides, DNA and/or RNA. The nucleic acid of interest introduced by the present method can be nucleic acid from any source, such as nucleic acid obtained from cells in which it occurs in nature, recombinantly produced nucleic acid or chemically synthesized nucleic acid. For example, the nucleic acid can be cDNA or 15 genomic DNA or DNA synthesized to have the nucleotide sequence corresponding to that of naturally-occurring DNA. The nucleic acid can also be a mutated or altered form of nucleic acid (e.g., DNA that differs from a naturally occurring DNA by an alteration, deletion, substitution or addition of at least one nucleic acid residue) or nucleic acid that does not occur in nature. Nucleic acid introduced by the 20 subject method can be present in a vector, such as an expression vector (e.g., a plasmid or viral-based vector), but it need not be. Nucleic acid of interest can be introduced into cells in such a manner that it becomes integrated into genomic DNA and is expressed or remains extrachromosomal (is expressed episomally). The nucleic acid for use in the methods of the present invention can be linear or circular 25 and can be of any size. For example, the nucleic acid can be from about 3 kb to about 10kb, from about 5 kb to about 8 kb and from about 6 kb to 7 kb. Nucleic acid introduced into cells by the method described herein can further comprise nucleic acid (e.g., DNA) that facilitates entry of the nucleic acid into cells or passage into the cell nucleus (nuclear localization elements).

30 The carrier for use in the methods of the present invention can be, for example, gelatin or an equivalent thereof.

Eukaryotic cells, such as mammalian cells (e.g., human, monkey, canine, feline, bovine, or murine cells), bacterial, insect or plant cells, are plated (placed) onto the surface bearing the DNA-containing mixture in sufficient density and under appropriate conditions for introduction/entry of the DNA into the eukaryotic cells

5 and expression of the DNA or its interaction with cellular components. Preferably, the eukaryotic cells (in an appropriate medium) are plated on top of the dried DNA-containing spots at high density (e.g.,  $0.5\text{--}1 \times 10^5/\text{cm}^2$ ), in order to increase the likelihood that reverse transfection will occur. For example, the density of cells can be from about  $0.3 \times 10^5/\text{cm}^2$  to about  $3 \times 10^5/\text{cm}^2$ , and in specific embodiments, is

10 from about  $0.5 \times 10^5/\text{cm}^2$  to about  $2 \times 10^5/\text{cm}^2$  and from about  $0.5 \times 10^5/\text{cm}^2$  to about  $1 \times 10^5/\text{cm}^2$ . The appropriate conditions for introduction/entry of DNA into cells will vary depending on the quantity of cells used.

Two embodiments of the present method are described in detail herein: a DNA-gelatin method, in which a mixture comprising DNA (e.g., DNA in an expression vector, such as, a plasmid-based or viral-based vector) and a carrier protein (e.g., gelatin) is used and a lipid vector-DNA method, in which a mixture comprising DNA, such as DNA in an expression vector (e.g., a plasmid); a carrier protein (e.g., gelatin); a sugar (e.g., sucrose); DNA condensation buffer; and an appropriate lipid-containing transfection reagent is used. Any suitable gelatin which

15 is non-toxic, hydrated, which can immobilize the nucleic acid mixture onto a surface and which also allows the nucleic acid immobilized on the surface to be introduced over time into cells plated on the surface can be used. For example, the gelatin can be a crude protein gelatin or a more pure protein based gelatin such as fibronectin. In addition, a hydrogel, a sugar based gelatin (polyethylene glycol) or a synthetic or

20 chemical based gelatin such as acrylamide can be used.

In the first embodiment, a mixture comprising two components (DNA such as DNA in an expression vector and a carrier protein) is spotted onto a surface (e.g., a slide) in discrete, defined locations or areas and allowed to dry. One example of this embodiment is described in Example 1. After the carrier (e.g., gelatin)-DNA mixture has dried sufficiently that it is affixed to the surface, transfection reagents (a lipofection mixture) and cells to be reverse transfected are added, preferably sequentially. The transfection mixture can be one made from available components

or can be a commercially available mixture, such as Effectene™ (Qiagen), Fugene™ 6 (Boehringer Mannheim) or Lipofectamine™ (Gibco/BRL-Life Technologies). It is added in an appropriate quantity, which can be determined empirically, taking into consideration the amount of DNA in each defined location. A wax barrier can be  
5 drawn around the locations on the surface which contain the vector-DNA mixture, prior to addition of the transfection mixture, in order to retain the mixture or the solution can be kept in place using a cover well. Generally, in this embodiment, the transfection reagent is removed, such as by vacuum suctioning, prior to addition of cells into which DNA is to be reverse transfected. Actively growing cells are plated  
10 on top of the locations, producing a surface that bears the DNA-containing mixture in defined locations. The resulting product is maintained under conditions (e.g., temperature and time) which result in entry of DNA in the DNA spots into the growing cells. These conditions will vary according to the types of cells and reagents used and can be determined empirically. Temperature can be, for example,  
15 room temperature or 37°C, 25°C or any temperature determined to be appropriate for the cells and reagents.

A variety of methods can be used to detect protein expression in the DNA-containing spots. For example, immunofluorescence can be used to detect a protein. Alternatively, expression of proteins that alter the phosphorylation state or  
20 subcellular localization of another protein, proteins that bind with other proteins or with nucleic acids or proteins with enzymatic activity can be detected.

In the second embodiment, one example of which is described in Example 2, a mixture comprising DNA in an expression vector; a carrier protein (e.g., gelatin); a sugar (e.g., sucrose); DNA condensation buffer; and a lipid-based transfection  
25 reagent is spotted onto a surface, such as a slide, in discrete, defined locations and allowed to dry. Actively growing cells are plated on top of the DNA-containing locations and the resulting surface is maintained under conditions (e.g., temperature and time) which result in entry of DNA in the DNA spots into the growing cells (reverse transfection). Expression of DNA in cells is detected using known  
30 methods, as described above.

Any suitable surface which can be used to affix the DNA containing mixture to its surface can be used. For example, the surface can be glass, polystyrene or plastic. In addition, the surface can be coated with, for example, polylysine.

The present invention also encompasses methods of making arrays which 5 comprise DNA affixed to a surface such that when cells are plated onto the surface bearing the DNA, the DNA can be introduced (is introducible) into the cells (i.e., the DNA can move from the surface into the cells). The present invention also encompasses a DNA array comprising a surface having affixed thereto, in discrete, defined locations, DNA of known sequence or source by a method described herein.

10 The methods of this invention are useful to identify DNAs of interest (DNAs that are expressed in recipient cells or act upon or interact with recipient cell constituents or function, such as DNAs that encode a protein whose function is desired because of characteristics its expression gives cells in which it is expressed). They can be used in a variety of formats, including macro-arrays and micro-arrays.

15 They permit a DNA array to be converted into a protein or cell array, such as a protein or cell microarray.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

#### Example 1 Reverse Transfection: "Gelatin-DNA" Method

##### 20 Materials

[DNA]: 1 $\mu$ g/ $\mu$ L (eg., HA-GST pRK5, pBABE CMV GFP)

Gelatin (ICN, cat.# 901771): 0.2% stock in ddH<sub>2</sub>O, all dilutions made in PBS

0.20% gelatin = 0.5g gelatin + 250mL ddH<sub>2</sub>O

Effectene Transfection Kit (Qiagen, cat.# 301425)

25 Plasmid-DNA: grown in 100mL L-amp overnight from glycerol stock, purified by standard Qiaprep Miniprep or Qiagen Plasmid Purification Maxi protocols

Cell Type: HEK 293T cultured in DMEM/10%IFS with L-glut and pen/strep

#### Diluting and Spotting DNA

- Dilute DNA in 0.2% gelatin\* to give final [DNA]=0.05 $\mu$ g/ $\mu$ L\*\*
- 30 • Spot DNA/gelatin mix on  $\Sigma$  poly-L-lysine slides using arrayer

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- Allow slides to dry in vacuum-dessicator overnight\*\*\*
  - \* range of gelatin concentration that worked under the conditions used = 0.05% to 0.5%
  - \*\* range of DNA concentrations that worked under the conditions used = 0.01  $\mu\text{g}/\mu\text{L}$  to 0.10  $\mu\text{g}/\mu\text{L}$
  - 5 \*\*\* range of drying time = 2 hours to 1 week

#### Adding Tx. Reagents to Gelatin -DNA Spots

- In eppendorf tube, mix 300  $\mu\text{L}$  DNA-condensation buffer (EC Buffer)+ 16  $\mu\text{L}$  Enhancer
- 10 • Mix by vortexing. Incubate for 5 minutes
- Add 50  $\mu\text{L}$  Effectene and mix by pipetting
- Draw a wax circular barrier on slide around spots to apply the transfection reagent
- Add 366  $\mu\text{L}$  mix to wax-enclosed region of spots
- 15 • Incubate at room temperature for 10 to 20 minutes
- Meanwhile, split cells to reverse-transfect
- Vacuum-suction off reagent in hood

Place slides in dish and add cells for reverse transfection

#### Splitting Cells

- 20 • Split actively growing cells to [cell] =  $10^7$  cells in 25mL
- Plate cells on top of slide(s) in square 100x100x15mm petri dish
- Allow reverse transfection to proceed for 40 hours = approx. 2 cell cycles
- Process slides for immunofluorescence

#### Example 2 Reverse Transfection: "Lipid - DNA" Method

##### 25 Materials

[DNA]:1  $\mu\text{g}/\mu\text{L}$  (eg., HA-GST pRK5, pBABE CMV GFP)

Gelatin (ICN, cat.# 901771): 0.2% stock in ddH<sub>2</sub>O, all dilutions made in PBS

0.05% gelatin = 250  $\mu\text{L}$  0.2% + 750  $\mu\text{L}$  PBS

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Effectene Transfection Kit (Qiagen, cat.# 301425):

EC Buffer in 0.4M sucrose = 273.6 $\mu$ L 50% sucrose + 726.4 $\mu$ L EC Buffer

Plasmid-DNA: grown in 100mL L-amp overnight from glycerol stock, purified by standard Qiaprep Miniprep or Qiagen Plasmid Purification Maxi protocols

5 Cell Type: HEK 293T cultured in DMEM/10%IFS with L-glut and pen/strep

Reverse Transfection Protocol with Reduced Volume

- Aliquot 1.6 $\mu$ g DNA in separate eppendorf tubes
- Add 15 $\mu$ L of pre-made *DNA-condensation buffer (EC Buffer) with 0.4M sucrose\** to tubes

10 • Add 1.6 $\mu$ L of Enhancer solution and mix by pipetting several times. Incubate at room temperature for 5 minutes

• Add 5 $\mu$ L of Effectene Transfection Reagent to the DNA-Enhancer mix and mix by pipetting. Incubate at room temperature for 10 minutes

• Add 23.2 $\mu$ L of 0.05% gelatin\*\* to total transfection reagent mix (i.e. 1:1 dilution)

• Spot lipid-DNA on  $\Sigma$  poly-L-lysine slides mix using arrayer

• Allow slides to dry in vacuum-dessicator overnight\*\*\*

Effectene™ kit (Qiagen) used includes Enhancer solution, which was used according to Qiagen's instructions.

20 \* range of sucrose that worked under the conditions used = 0.1M to 0.4M

\*\* range of gelatin concentration that worked under the conditions used = 0.01% to 0.05%

\*\*\* range of drying time = 2 hours to 1 week

Splitting Cells

25 • Split actively growing cells to [cell] =  $10^7$  cells in 25mL

• Plate cells on top of slide(s) in square 100x100x15mm petri dish

• Allow reverse transfection to proceed for 40 hours = approx. 2 cell cycles

• Process slides for immunofluorescence

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Example 3 Transfected Cells Micorarrays: a genomics approach for the analysis of gene products in mammalian cells

Lipid-DNA Method

I. Gelatin Preparation and DNA Purification

5 Materials:

Gamma-Amino Propyl Silane (GAPS) slides (Corning catalog #2550),  
Purified cDNA,  
Gelatin, Type B: 225 Bloom (Sigma, catalog #G-9391),

Methods:

10 0.2% Gelatin was made by incubation in a 60°C water bath for 15 minutes. The gelatin was cooled slowly to 37°C at which point it was filtered through 0.45µm cellular acetate membrane (CA).

Bacterial clones with DNA plasmids were grown in a 96 Deep-Well Dish for 18 to 24 hours in 1.3mL of terrific broth (TB) shaking at 250rpm at 37°C. The plasmids  
15 were miniprepped and optical density (OD) was taken. DNA purity, as indicated by final 280nm/260nm absorbance ratio, was greater than 1.7.

Storage:

For storage purposes, gelatin was kept at 4°C and miniprepped DNA kept at -20°C.

II. Sample Preparation and Array Printing

20 Materials:

Effectene Transfection Reagent (Qiagen catalog #301425),  
Sucrose (Life Technologies),  
INTEGRID 100mm x 15mm Tissue Culture Square Petri Dishes (Becton Dickinson:  
Falcon catalog #35-1012),  
25 Costar 384-well plates (VWR catalog #7402),  
Stealth Micro Spotting Pins, (Telechem International, Inc. catalog #SMP4),  
PixSys 5500 Robotic Arrayer (Cartesian Technologies, Model AD20A5),

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Vacuum Dessicator with Stopcock 250mm, NALGENE (VWR catalog #24987-004),

DRIERITE Anhydrous Calcium Sulfate (VWR catalog #22890-229)

Forceps to hold slides,

- 5 Human Embryonic Kidney (HEK) 293T cells,  
Tissue Culture hood,  
Cover Slips (50mm x 25mm),

Methods:

For each DNA-containing spot, 15 $\mu$ l of pre-made DNA-condensation buffer (Buffer

- 10 EC) with 0.2M to 0.4M sucrose was added to 0.80 $\mu$ g to 1.60 $\mu$ g DNA in a separate eppendorf tube. Subsequently, 1.5 $\mu$ l of the Enhancer solution was added to the tube and mixed by pipetting. This was let to incubate at room temperature for 5 minutes. 5 $\mu$ l Effectene transfection reagent was added, mixed and let to incubate at room temperature for 10 minutes with the DNA-Enhancer mixture. 1X volume of 0.05% gelatin was added, mixed and the appropriate amount was aliquoted into a 384-well plate for arraying purposes.

The PixSys 5500 Robotic Arrayer was used with Telechem's ArrayIt Stealth Pins (SMP4) with each spot spaced 400 $\mu$ m apart with a 50ms to 500ms delay time of the pin on the slide for each spot. A 55% relative humidity environment was

- 20 maintained during the arraying. A thorough wash step was implemented between each dip into a DNA sample in the 384-well plate to avoid clogging of the pins that would result in missing spots in the array.

In a tissue culture hood, 10x10<sup>6</sup> Human Embryonic Kidney (HEK) 293T cells were prepared in 25ml DME media with 10% IFS, pen/strep and glutamine for every 3

- 25 slides that were to be processed. After arraying, the slides were simply placed array-side facing up on a sterile 100x100x10mm square dish (3 slides per plate) and the cells were poured gently on the slides while avoiding direct pouring on the arrays themselves. If the number of slides were not a multiple of 3, dummy slides were placed to cover the square dish.

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The cells were let to grow on the arrays for approximately 2 cell cycles (~40hours for 293T). Subsequently, the slides were very gently rinsed with PBS<sup>-</sup> in a Coplin jar, and then fixed in 3.7% paraformaldehyde/4.0% sucrose for 20 minutes in a Coplin jar, and then transferred back to a jar with PBS<sup>-</sup>.

5 Storage:

After arraying, slides were stored at room temperature in a vacuum dessicator with anhydrous calcium sulfate pellets. After fixation, slides were kept in PBS<sup>-</sup> at 4°C until analyses were completed (maximum of 5 days).

III. Methods of Detection

10 Immunofluorescence

Fluorescence Microscopy

Laser Scanning

Radiolabelling and detection with sensitive film or emulsion

If the expressed proteins to be visualized are fluorescent proteins, they can be

15 viewed and photographed by fluorescent microscopy. For large expression array, slides may be scanned with a laser scanner for data storage. If a fluorescent antibody can detect the expressed proteins, the protocol for immunofluorescence can be followed. If the detection is based on radioactivity, the slides can be fixed as indicated above and radioactivity detected by autoradiography with film or  
20 emulsion.

Immunofluorescence:

After fixation, the array area was permeabilized in 0.1% Triton X-100 in PBS<sup>-</sup> for 15 minutes. After two rinses in PBS<sup>-</sup>, the slides were blocked for 60 minutes, probed with a primary antibody at 1:200 to 1:500 dilution for 60 minutes, blocked for 20

25 minutes, probed with a fluorescent secondary antibody at 1:200 dilution for 40 minutes. The slides can be transferred to a Coplin jar in PBS<sup>-</sup> and visualized under an upright fluorescent microscope. After analyses, the slides can be mounted and stored in the dark at 4°C.

To create these microarrays, distinct and defined areas of a lawn of cells were simultaneously transfected with different plasmid DNAs (Figure 4A). This is accomplished without the use of individual wells to sequester the DNAs. Nanoliter volumes of plasmid DNA in an aqueous gelatin solution are printed on a glass slide.

5 A robotic arrayer (PixSys 5500, Cartesian Technologies) equipped with stealth pins (SMP4, Telechem) was used to print a plasmid DNA/gelatin solution contained in a 384-well plate onto CMT GAPS glass slides (Corning). The pins deposited ~1 nl volumes 400  $\mu$ m apart using a 25 ms pin down slide time in a 55% relative humidity environment. Printed slides were stored at room temperature in a vacuum desiccator

10 until use. Preparation of aqueous gelatin solution is important and is as follows. 0.02% gelatin (w/v) (Sigma G-9391) was dissolved in MilliQ water by heating and gentle swirling in a 60°C water bath for 15 minutes. The solution was cooled slowly to room temperature and filtered through a 0.45 $\mu$ m cellular acetate membrane and stored at 4°C. Plasmid DNA was purified with the Plasmid Maxi or QIAprep 96

15 Turbo Miniprep kits (Qiagen), and always had an A260/A280>1.7. Concentrated solutions of DNA were diluted in the gelatin solution so to keep the gelatin concentration >0.017% and, unless otherwise specified, final plasmid DNA concentrations were 0.033  $\mu$ g/ $\mu$ l. To express GFP the EGFP construct in pBABEpuro was used.

20 After drying, the DNA spots are briefly exposed to a lipid transfection reagent, the slide is placed in a culture dish and covered with adherent mammalian cells in media. The Effectene transfection kit (301425, Qiagen) was used as follows. In a 1.5 ml microcentrifuge tube, 16  $\mu$ l enhancer was added to 150  $\mu$ l EC buffer, mixed, and incubated for 5 minutes at room temperature. 25  $\mu$ l effectene lipid was

25 added, mixed and the entire volume pipetted onto a 40 x 20 mm cover well (PC200, Grace Bio-Labs). A slide with the printed side down was placed on the cover well such that the solution covers the entire arrayed area while also creating an airtight seal. After a 10 minute incubation, the cover well was pried off the slide with a forceps and the transfection reagent removed carefully by vacuum aspiration. The

30 slide was placed printed side up in a 100 x 100 x 10 mm square tissue culture dish and a  $1 \times 10^7$  actively growing HEK293T cells in 25 ml media (DMEM with 10% FBS, 50 units/ml penicillin and 50  $\mu$ g/ml streptomycin) were poured into the dish.

Three slides can be transfected side-by-side in this fashion. The cells grew on the slide for 40 hours prior to fixing for 20 minutes at room temperature in 3.7% paraformaldehyde/4.0% sucrose in PBS. Other commonly used mammalian cell lines, such as HeLa and A549 cells, were also tested and similar results were obtained but with transfection efficiencies of 30-50% of those obtained with HEK293 cells. The DNA in the gelatin gel is insoluble in cell culture media but readily enters cells growing on it to create the transfected cell microarray.

To illustrate the method, an array with elements containing an expression construct for the green fluorescent protein (GFP) was printed. HEK293 cells were plated on the slide for transfection and the fluorescence of the cells detected with a laser fluorescence scanner. Microarrays were imaged at a resolution of 5 $\mu$ m with a laser fluorescence scanner (ScanArray 5000, GSI Lumonics). GFP and cy3 emission was measured separately after sequential excitation of the two fluorophores. To obtain images at cellular resolution, cells were photographed with a conventional fluorescent microscope. All images were pseudocolored and superimposed using Photoshop 5.5 (Adobe Systems).

A low magnification scan showed a regular pattern of fluorescent spots that matches the pattern in which the GFP expression construct was printed (Figure 4B). A higher magnification image obtained via fluorescence microscopy showed that each spot is about 150  $\mu$ m in diameter and consists of a cluster of 30-80 fluorescent cells (Figure 4C). As in a conventional transfection, the total expression level in the clusters is proportional over a defined range to the amount of plasmid DNA used (Figure 4D). Since it may be useful to express two different plasmids in the same cells, whether the technique is compatible with cotransfection was examined.

Arrays with elements containing expression constructs for GFP, an epitope-tagged protein or both were prepared and transfected. The cells growing on elements printed with both cDNAs express both encoded proteins, indicating that cotransfection had occurred (Figure 4E).

Whether transfected cell microarrays could be used to clone gene products based on their intrinsic properties was also determined. As a test case, an array to identify the receptor for FK506, a clinically important immunosuppressant whose pharmacologically relevant target, FKBP12, is an intra-cellular protein, was used

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(Kino, T., et al., *J. Antimicrob.*, 40:1256 (1987); Harding, M.W., et al., *Nature*, 26:755 (1989)). Elements containing expression constructs for FKBP12, GFP, or both were printed on a slide, in an easily recognizable pattern. After the transfected cell microarray formed, radiolabeled FK506 was added to the tissue culture media  
5 for one hour prior to processing the slide for autoradiography and immunofluorescence. The radiolabeled FK506 bound to the array in a pattern of spots that exactly matches the pattern of cell clusters expressing FKBP12 (Figure 5A). Detection of the bound FK506 with autoradiographic emulsion confirmed, at the cellular level, colocalization between FKBP12 expression and FK506 binding  
10 (Figure 5B). The binding is specific because the GFP-expressing clusters and the non-transfected cells surrounding the clusters showed only background levels of signal (Figure 5A). Furthermore, the prior addition of excess rapamycin, a competitive antagonist of FK506, completely eliminated the signal. 1  $\mu$ M rapamycin was added to the cell culture media 30 minutes before the addition of  
15 radiolabeled FK506.

The utility of transfected cell microarrays for identifying gene products that induce phenotypes of interest in mammalian cells or have a distinct sub-cellular localization was also explored. Arrays with a collection, enriched for signaling molecules, of 192 distinct epitope-tagged cDNAs in expression vectors were printed.  
20 192 Genestrom expression constructs (Invitrogen) in bacteria were cultured in two 96-well plates and plasmid DNA was purified using the Turbo Miniprep Kit (Qiagen). Plasmid DNA was diluted with 0.02% gelatin to a final concentration of 0.040  $\mu$ g/ $\mu$ l and printed. Cellular phosphotyrosine levels were determined by immunofluorescence staining and scanning. Cell morphology and subcellular  
25 localization of expressed proteins was assessed by visual inspection via fluorescence microscopy of the cells in the clusters after their detection with anti-V5 immunofluorescence.

After transfection, their effects on cellular phosphotyrosine levels and morphology as well as their subcellular localization were determined. Five cell  
30 clusters on grid 1 (A2, C7, C9, C11, and F6) had phosphotyrosine levels above background (Figure 5C). The coordinates of the clusters match those of the wells of a microtiter plate containing the source cDNAs and were used to look up the identity

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of the transfected cDNAs. This revealed that four of these clusters were transfected with known tyrosine kinases (trkC, syk, syn, and blk) while the fifth (C11) encodes a protein of unknown function. Simple visual examination of the morphology of the cells in the transfected clusters revealed a diversity of cellular phenotypes even in  
5 this small set of clones. In array 2, cluster E8 had fragmented cells characteristic of apoptosis while in two clusters (D10 and F7) the cells were closely attached to each other (Figure 5D). The presence of apoptotic cells was confirmed by TUNEL (Terminal deoxynucleotidyl transferase mediated d<sub>UTP</sub>-biotin nick end labeling method) staining. TUNEL staining was performed as described (Y. Gavrieli, Y.  
10 Sherman, S.A. Ben-Sasson. J. Cell Biol. 119, 493 (1992)).

The observed phenotypes are consistent with the presumed functions of the cDNAs expressed in these clusters (the Table). Subcellular localization of the expressed proteins were examined through visual inspection the and those with distinct patterns were noted (the Table). This revealed that several proteins that are  
15 known transcription factors were mainly located in the cell nucleus. This was also true for other proteins, such as phosphatase 1-beta, whose subcellular distribution has not been previously ascertained.

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## TABLE

Description of selected cDNAs expressed in the transfected cell microarray. Shown are the coordinates, the phenotype or property detected, the Genbank accession number and the name of the cDNA. nuc/cyo means nuclear and cytoplasmic staining was visible.

	Grid: Coordinate	Phenotype/property	Accession number	Function
5	2:E8	apoptosis	AF016266	TRAIL receptor 2
	2:D10	cell adhesion	X97229	NK receptor
	2:F7	cell adhesion	M98399	CD36
10	1:A9	nuclear	U11791	Cyclin H
	1:B5	nuclear	M60527	deoxycytidine kinase
	1:B12	nuclear	M60724	p70 S6 kinase kinase $\alpha$ 1
	1:C12	nuclear	M90813	D-type cyclin
	1:E4	mitochondrial	U54645	methylmalonyl-coA mutase
15	1:E10	mitochondrial	J05401	creatine kinase
	1:G9	nuc/cyo	U40989	tat interactive protein
	1:G10	nuc/cyo	U09578	MAPKAP (3pk) kinase
	2:A9	nuclear	X83928	TFIID subunit TAFII28
	2:A12	nuc/cyo	M62831	ETR101
20	2:B6	nuc/cyo	X06948	IgE receptor $\alpha$ -subunit
	2:B12	nuclear	X63469	TFIIE $\beta$ subunit
	2:C5	nuclear	M76766	General transcription factor IIB
	2:C7	nuc/cyo	M15059	CD23A
	2:C12	nuclear	X80910	PP1, $\beta$ catalytic subunit
25	2:D4	nuclear	AF017307	Ets-related transcription factor
	2:E7	nuclear	X63468	TFIIE $\alpha$
	2:E12	nuclear	U22662	Orphan receptor LXR- $\alpha$
	2:F8	nuclear	L08895	MEF2C
	2:F12	nuclear	AF028008	SP1-like transcription factor
30	2:G2	nuc/cyo	U37352	PP2A, regulatory B' $\alpha$ 1 subunit
	2:G3	nuc/cyo	L14778	PP2B, catalytic $\alpha$ subunit

The microarrays can be printed with the same robotic arrayers as traditional DNA arrays, so it is feasible to achieve densities of up 10,000-15,000 cell clusters

per standard slide. At these densities the entire set of human genes can be expressed on a small number of slides, allowing rapid pan-genomic screens. Thus, comprehensive collections of full-length cDNAs for all mammalian genes can be generated (Strausberg, R.L., *et al.*, *Science*, 15:455 (1999);

5 [www.hip.harvard.edu/research.html](http://www.hip.harvard.edu/research.html), [www.guthrie.org/cDNA](http://www.guthrie.org/cDNA)) and will be valuable tools for making such arrays.

Transfected cell microarrays have distinct advantages over conventional expression cloning strategies using FACs or sib selection (Simonsen, H., *et al.*, *Trends Pharmacol. Sci.*, 15:437 (1994)). First, cDNAs do not need to be isolated  
10 from the cells exhibiting the phenotype of interest. This allows for screens using a variety of detection methods, such as autoradiography or *in situ* hybridization, and significantly accelerates the pace of expression cloning. The experiments described herein took days to perform instead of the weeks to months necessary with other expression cloning strategies. Second, transfected cell microarrays can also be used  
15 to screen living cells, allowing the detection of transient phenotypes, such as changes in intracellular calcium concentrations. Third, being compact and easy to handle, transfected cell microarrays have economies of scale. The arrays are stable for months and can be printed in large numbers, allowing many phenotypes to be screened in parallel, with a variety of methods, in a small number of tissue culture  
20 plates.

Described herein are arrays in which the transfected plasmids direct gene overexpression. However, as antisense technology improves or other methods emerge for decreasing gene function in mammalian cells, it is likely that transfected cell microarrays can be used to screen for phenotypes caused by loss of gene  
25 function. Lastly, the immobilization of the plasmid DNA in a degradable gel is the key to spatially restricting transfection without wells.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without  
30 departing from the scope of the invention encompassed by the appended claims.

## CLAIMS

What is claimed is:

1. A reverse-transfection method of introducing DNA into eukaryotic cells comprising:
  - 5 (a) depositing a DNA-containing mixture onto a surface in discrete, defined locations, wherein the DNA-containing mixture comprises DNA to be introduced into the eukaryotic cells and a carrier protein and allowing the DNA-containing mixture to dry on the surface, thereby producing a surface having the DNA-containing mixture affixed thereon in discrete, defined locations; and
  - (b) plating the eukaryotic cells onto the surface in sufficient density and under appropriate conditions for entry of DNA in the DNA-containing mixture into eukaryotic cells, whereby DNA in the DNA-containing mixture is introduced into the eukaryotic cells.
- 15 2. The method of claim 1, wherein the DNA to be introduced is contained in a vector; the carrier protein is gelatin; the slide is a glass slide or a Σ poly-L-lysine slide and the eukaryotic cells are mammalian cells.
- 20 3. The method of claim 2, wherein the vector is a plasmid or a viral-based vector.
4. The method of claim 2, wherein the gelatin concentration in the DNA-containing mixture is from about 0.05% to about 0.5%
- 25 5. A method of introducing DNA of interest into eukaryotic cells, comprising:
  - (a) depositing a carrier-DNA mixture onto a surface in discrete, defined locations, wherein the carrier-DNA mixture comprises DNA of interest and a carrier protein, and allowing the carrier-DNA mixture to dry on

the surface, thereby producing a surface bearing the carrier-DNA mixture in discrete defined locations;

(b) covering the surface bearing the carrier-DNA mixture with an appropriate amount of a lipid-based transfection reagent and  
5 maintaining the resulting product under conditions appropriate for complex formation between DNA in the carrier-DNA mixture and the transfection reagent;

(c) removing transfection reagent, thereby producing a surface bearing DNA;  
10 (d) plating the eukaryotic cells onto the surface bearing DNA, in sufficient density and under appropriate conditions for entry of the DNA into the eukaryotic cells,  
whereby DNA of interest is introduced into the cells.

15 6. The method of claim 5, wherein the carrier protein is gelatin and the surface is the surface of a slide.

7. The method of claim 6, wherein the slide is a glass slide or a  $\Sigma$  poly-L-lysine slide.

20 8. The method of claim 7, wherein the concentration of gelatin in the vector-DNA mixture is from about 0.05% to about 0.5%.

9. The method of claim 8, wherein the concentration of gelatin is from about 0.1% to about 0.2%.

25 10. The method of claim 5, wherein the DNA of interest is in an expression vector and eukaryotic cells that contain DNA of interest are maintained under conditions appropriate for expression of the DNA, whereby DNA of interest is expressed.

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11. The method of claim 10, further comprising identifying eukaryotic cells in which a protein of interest is expressed, comprising contacting eukaryotic cells on the surface with an antibody which binds the protein of interest and detecting binding of the antibody, wherein binding identifies eukaryotic cells  
5 in which the protein of interest is expressed.
12. A method of introducing DNA of interest into eukaryotic cells, comprising:
  - (a) depositing a gelatin-DNA mixture onto a surface in discrete, defined locations, wherein the gelatin-DNA mixture comprises DNA of interest and a gelatin, and allowing the gelatin-DNA mixture to dry on the surface, thereby producing a surface bearing the gelatin-DNA mixture in discrete defined locations;
  - (b) covering the surface bearing the gelatin-DNA mixture with an appropriate amount of a lipid-based transfection reagent and maintaining the resulting product under conditions appropriate for complex formation between DNA in the gelatin-DNA mixture and the transfection reagent;
  - (c) removing transfection reagent, thereby producing a surface bearing DNA;
  - (d) plating the eukaryotic cells onto the surface bearing DNA, in sufficient density and under appropriate conditions for entry of the DNA into the eukaryotic cells,  
20 whereby DNA of interest is introduced into the cells.
13. The method of claim 12, wherein the surface is the surface of a slide.
- 25 14. The method of claim 13, wherein the slide is a glass slide or a Σ poly-L-lysine slide.
15. The method of claim 14, wherein the concentration of gelatin in the vector-DNA mixture is from about 0.05% to about 0.5%.

16. The method of claim 15, wherein the concentration of gelatin is from about 0.1% to about 0.2%.

17. The method of claim 12, wherein the DNA of interest is in an expression vector and eukaryotic cells that contain DNA of interest are maintained under conditions appropriate for expression of the DNA, whereby DNA of interest is expressed.

18. The method of claim 17, further comprising identifying eukaryotic cells in which a protein of interest is expressed, comprising contacting eukaryotic cells on the surface with an antibody which binds the protein of interest and detecting binding of the antibody, wherein binding identifies eukaryotic cells in which the protein of interest is expressed.

19. The method of claim 4, wherein the eukaryotic cells are mammalian cells and are plated in (b) at high density onto the surface bearing the vector-DNA mixture.

20. A method of introducing DNA of interest into eukaryotic cells, comprising:  
(a) depositing a lipid-DNA mixture onto a surface in discrete, defined locations, wherein the lipid-DNA mixture comprises DNA of interest; a carrier protein; a sugar; a buffer that facilitates DNA condensation and an appropriate lipid-based transfection reagent and allowing the lipid-DNA mixture to dry on the surface, thereby producing a surface bearing the lipid-DNA mixture in defined locations;  
(b) plating the eukaryotic cells onto the surface bearing the lipid-DNA mixture in sufficient density and under appropriate conditions for entry of DNA of interest into the eukaryotic cells,  
whereby DNA of interest is introduced into the cells.

21. The method of claim 20, wherein the carrier protein is gelatin and the surface is the surface of a slide.

22. The method of claim 21, wherein the slide is a glass slide or a  $\Sigma$  poly-L-lysine slide.
- 5 23. The method of claim 22, wherein the concentration of gelatin in the lipid-DNA mixture is from about 0.01% to about 0.05% and the concentration of sucrose is from about 0.1M to about 0.4M.
- 10 24. The method of claim 20, wherein the DNA of interest is in an expression vector and eukaryotic cells that contain DNA of interest are maintained under conditions appropriate for expression of the DNA, whereby DNA of interest is expressed.
- 15 25. A method of affixing DNA to a surface, to produce an array of DNA in discrete, defined locations of known sequence or source, comprising spotting of carrier-DNA mixture onto the surface in discrete, defined locations and allowing the resulting surface bearing the carrier-DNA mixture to dry sufficiently that the spots, referred to as DNA-containing spots, remain affixed to the surface under conditions in which the arrays are used.
- 20 26. A method of affixing DNA to a surface, to produce an array of DNA in discrete, defined locations of known sequence or source, comprising spotting of gelatin-DNA mixture onto the surface in discrete, defined locations and allowing the resulting surface bearing the gelatin-DNA mixture to dry sufficiently that the spots, referred to as DNA-containing spots, remain affixed to the surface under conditions in which the arrays are used.
- 25 27. A method of affixing DNA to a surface, to produce an array of DNA in discrete, defined locations of known sequence or source, comprising spotting a lipid-DNA mixture onto the surface in discrete, defined locations to produce spots and allowing the resulting surface bearing the lipid-DNA

mixture to dry sufficiently that the spots remain affixed to the surface under conditions in which the arrays are used.

28. A method of producing an array on a surface of reverse transfected cells that contain defined DNA, comprising:

5       a) spotting a carrier-DNA mixture spotting of gelatin-DNA mixture onto the surface in discrete, defined locations and allowing the resulting surface bearing the carrier-DNA mixture to dry sufficiently that the spots, referred to as DNA-containing spots, remain affixed to the surface under conditions in which the arrays are used;

10      b) covering the surface bearing the DNA-containing spots with an appropriate amount of a lipid-based transfection reagent and maintaining the resulting product under conditions appropriate for complex formation between DNA in the spots and the transfection reagent;

15      c) removing transfection reagent, producing a surface bearing DNA;

        d) adding cells in an appropriate medium to the surface bearing DNA, to produce a surface bearing DNA and plated cells; and

        e) maintaining the surface bearing DNA and plated cells under conditions that result in entry of DNA into plated cells, thus producing an array of

20      reverse transfected cells that contain defined DNA.

29. A method of producing an array on a surface of reverse transfected cells that contain defined DNA, comprising:

25      a) spotting a gelatin-DNA mixture spotting of gelatin-DNA mixture onto the surface in discrete, defined locations and allowing the resulting surface bearing the gelatin-DNA mixture to dry sufficiently that the spots, referred to as DNA-containing spots, remain affixed to the surface under conditions in which the arrays are used;

        b) covering the surface bearing the DNA-containing spots with an appropriate amount of a lipid-based transfection reagent and maintaining the resulting product under conditions appropriate for

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complex formation between DNA in the spots and the transfection reagent;

- 5           c) removing transfection reagent, producing a surface bearing DNA;
- d) adding cells in an appropriate medium to the surface bearing DNA, to produce a surface bearing DNA and plated cells; and
- e) maintaining the surface bearing DNA and plated cells under conditions that result in entry of DNA into plated cells, thus producing an array of reverse transfected cells that contain defined DNA.

10          30. A method of producing on a surface an array of reverse transfected cells that contain defined DNA, comprising:

- a) spotting a lipid-DNA mixture onto the surface in discrete, defined locations, to produce spots and allowing the resulting surface bearing the lipid-DNA mixture to dry sufficiently that the spots remain affixed to the surface under conditions in which the arrays are used;
- 15         b) plating cells on top of the surface produced in (a) and maintaining the resulting surface, which contains dried lipid-DNA mixture and cells to be reverse transfected, under conditions appropriate for growth of cells and entry of DNA into cells, thus producing an array of reverse transfected cells.

20          31. An array produced by the method of Claim 25

         32. An array produced by the method of Claim 26.

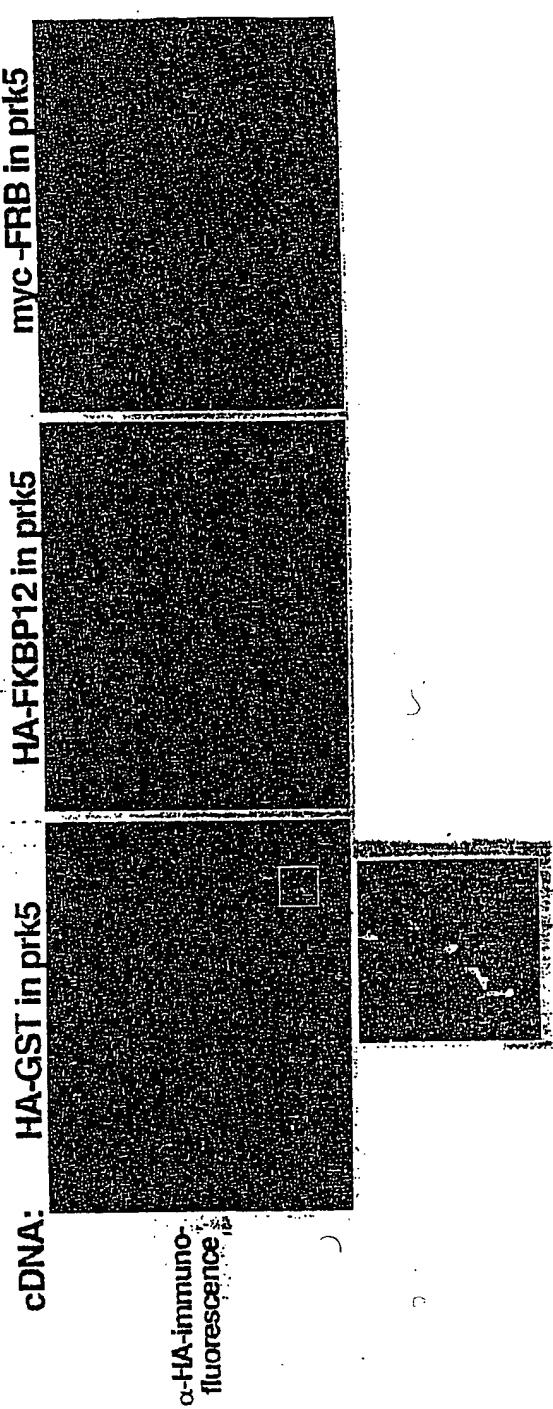
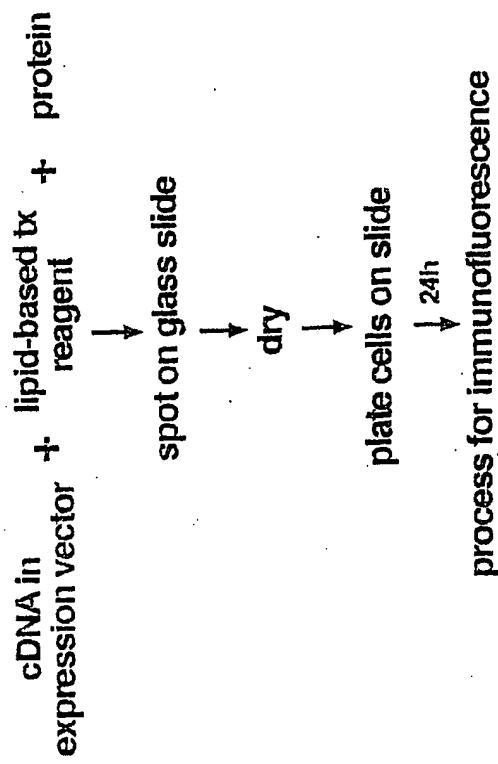
         33. An array produced by the method of Claim 27.

         34. An array produced by the method of Claim 28.

         35. An array produced by the method of Claim 29.

25          36. An array produced by the method of Claim 30.

## Reverse Transfections



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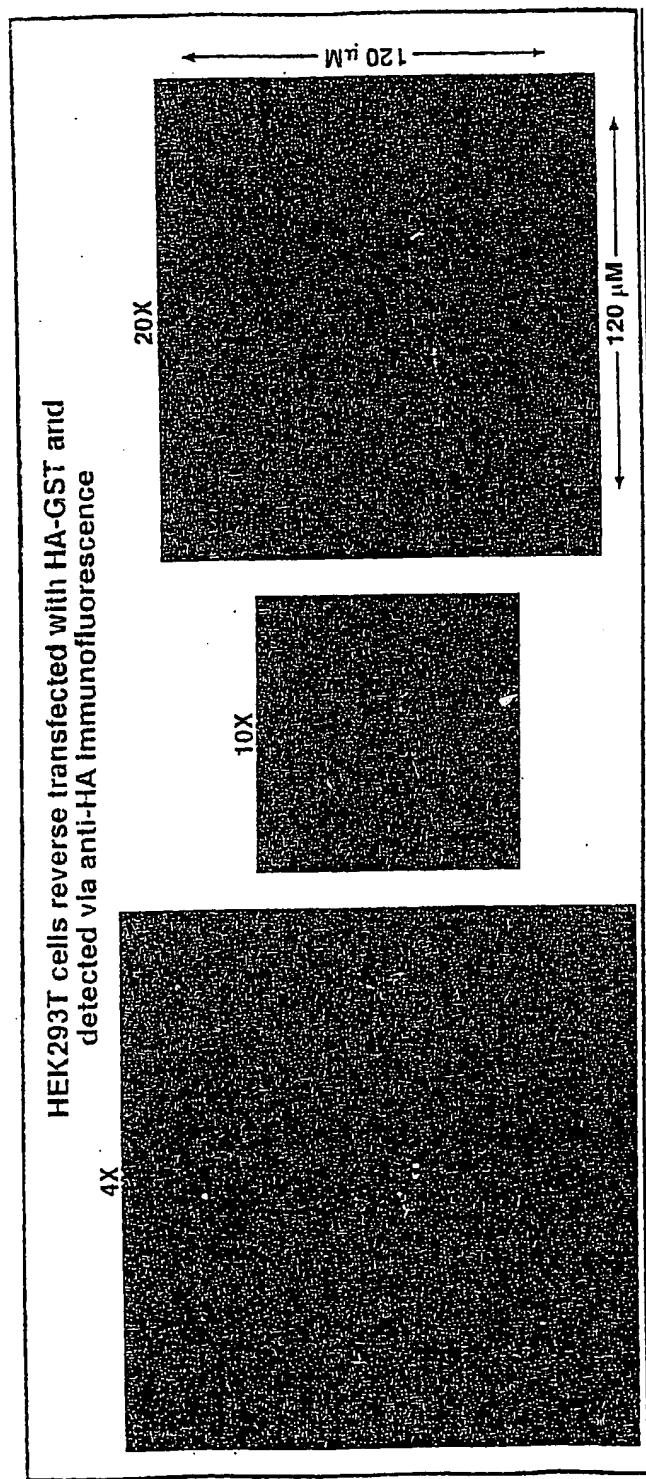


Figure 2

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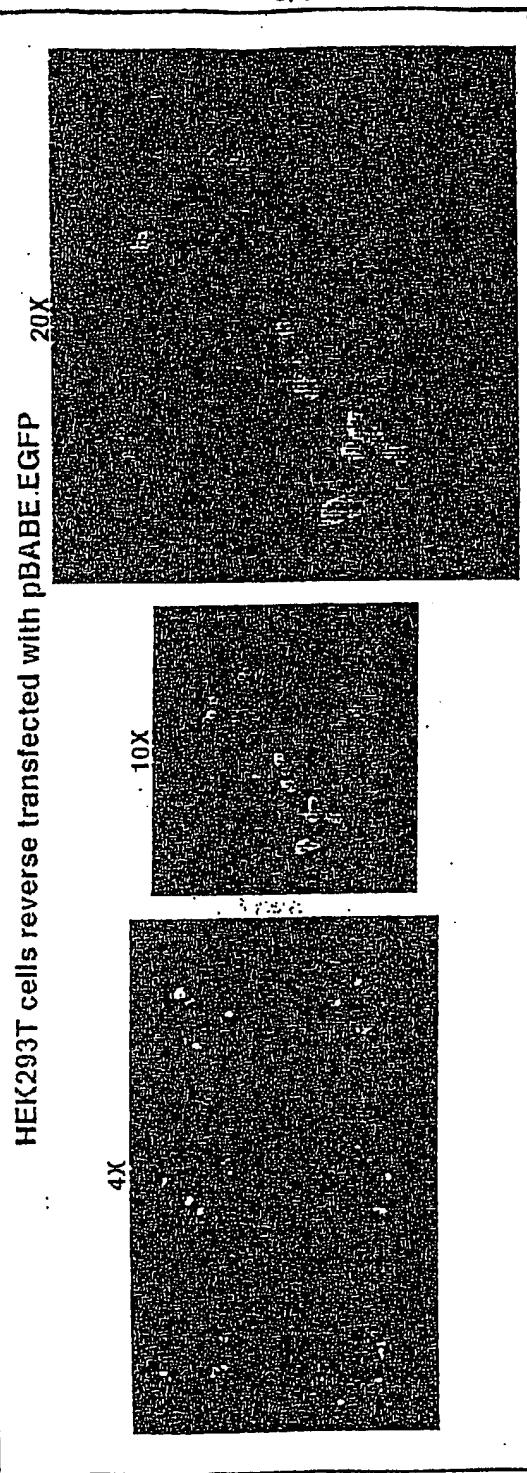
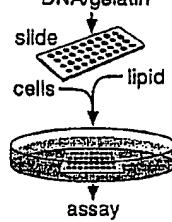
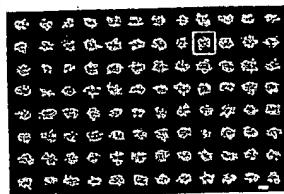
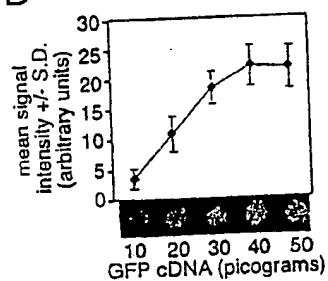
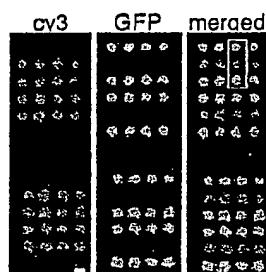


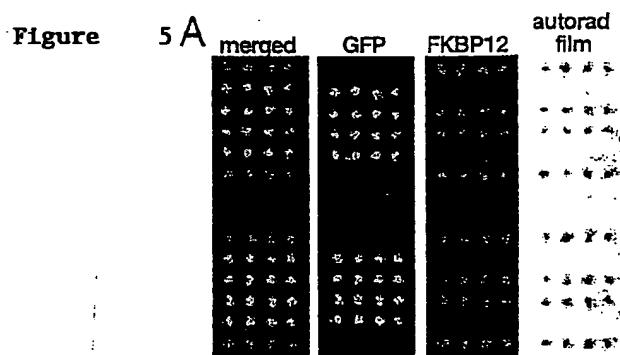
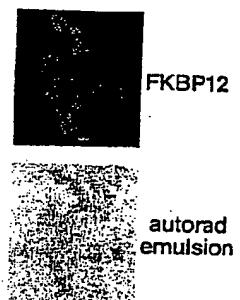
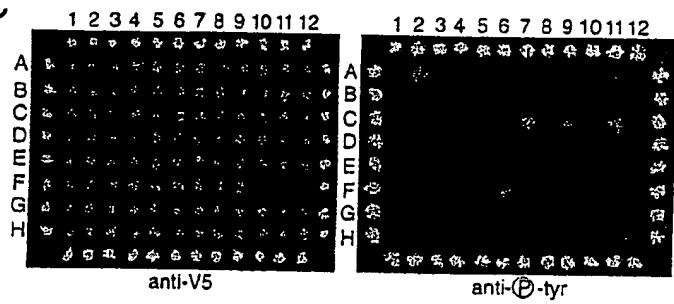
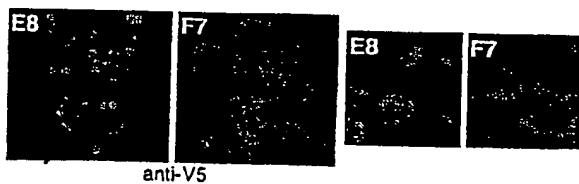
Figure 3

**Figure 4 A****Figure 4 B****Figure 4 C**

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**Figure 4 D****Figure 4 E****Figure 4 F**

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**Figure 5 B****Figure 5 C****Figure 5 D**

# INTERNATIONAL SEARCH REPORT

Inten [redacted] Application No  
PCT/US 00/25457

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C12N15/88 C12N15/10 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 99 55886 A (GENOVA PHARMACEUTICALS CORP) 4 November 1999 (1999-11-04) page 19 -page 20	1
X	WO 95 35505 A (UNIV LELAND STANFORD JUNIOR) 28 December 1995 (1995-12-28) the whole document	25
A	WO 96 17948 A (UNIV MICHIGAN) 13 June 1996 (1996-06-13) page 5, line 29 -page 6, line 5	1-36
A	US 5 851 818 A (OTO EDWIN KIYOSHI ET AL) 22 December 1998 (1998-12-22) abstract column 5, line 28 - line 40	1-36

Further documents are listed in continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

9 January 2001

Date of mailing of the international search report

17/01/2001

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## INTERNATIONAL SEARCH REPORT

Int'l Application No

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Patent document cited in search report		Publication date		Patent family member(s)		Publication date
WO 9955886	A	04-11-1999	AU	3572799 A		16-11-1999
WO 9535505	A	28-12-1995	US	5807522 A		15-09-1998
			AT	180570 T		15-06-1999
			AU	709276 B		26-08-1999
			AU	2862995 A		15-01-1996
			CA	2192095 A		28-12-1995
			DE	69509925 D		01-07-1999
			DE	69509925 T		09-12-1999
			DK	804731 T		08-11-1999
			EP	0804731 A		05-11-1997
			EP	0913485 A		06-05-1999
			ES	2134481 T		01-10-1999
			GR	3030430 T		30-09-1999
			JP	10503841 T		07-04-1998
			US	6110426 A		29-08-2000
WO 9617948	A	13-06-1996	US	5811274 A		22-09-1998
			CA	2206600 A		13-06-1996
			EP	0797680 A		01-10-1997
			JP	10510156 T		06-10-1998
			US	5654185 A		05-08-1997
			US	5804431 A		08-09-1998
US 5851818	A	22-12-1998	WO	0015825 A		23-03-2000
			AU	9568698 A		03-04-2000
			US	6133026 A		17-10-2000